OSTEOCLASTGENIC INHIBITORY AGENT

Background of the Invention

Field of the Invention

The present invention relates to an osteoclastgenic inhibitory agent comprising an interleukin-18 (hereinafter abbreviated as "IL-18") or its functional equivalent.

Description of the Prior Art

Osteoblasts' bone formation and osteoclasts' bone resorption are well balanced in healthy living bodies, and this keeps the bone tissues in normal conditions while old bone tissues are being replaced with fresh ones without altering the original bone shape. The phenomenon plays an important role in keeping living bodies' homeostasis such as the controlling of blood calcium concentration within a desired range. Once the balance is lost, especially when the bone resorption level exceeds the bone formation level, bone-related diseases and other diseases may be induced. Therefore, elucidation of the whole mechanism of bone resorption in living bodies, particularly, elucidation of osteoclasts is greatly highlighted due to scientific and clinical significance thereof.

However, the mechanism of osteoclast formation has not yet been completely elucidated even though interleukin 1 as a promoter and interleukin 4 as an inhibitor were found. This is because, similarly as various phenomena in living bodies, osteoclast formation in living bodies is controlled by the close and complicated relationship between promoters and inhibitors.

Based on these, it is greatly expected to establish an effective osteoclastgenic inhibitory agent from the viewpoint of scientific and clinical aspects.

Summary of the Invention

The object of the present invention is to provide a novel and effective osteoclastgenic inhibitory agent. To solve the object the present inventors energetically studied for IL-18, i.e., one of cytokines as communication transferring substances in immune systems, which induces production of interferon-γ (hereinafter abbreviated as "**IFN-γ**"), an important biologically active substance for immunocompetent cells, and granulocyte/macrophage colony-stimulating factor (hereinafter abbreviated as "**GM-CSF**"), and augments cytotoxicity and induces formation of killer cells. At the finding, IL-18 was described as an **interferon-γ-inducing factor** as reported by Haruki OKAMURA in Japanese Patent Kokai Nos. 27,189/96 and 193,098/96, and in *Nature*, Vol. 378, No. 6,552, pp. 88-91 (1995), and then called **IL-18** according to the proposal of Shimpei USHIO et al., in *The Journal of Immunology*, Vol. 156, pp. 4,274-4,279 (1996).

The present inventors found that a particular gene, capable of inhibiting osteoclast formation from osteoclastic precursor cells *in vitro*, is specifically expressed in quantities in stroma cells derived from mouse myeloma. Their further detailed analysis revealed that (i) the gene encodes IL-18 that includes SEQ ID NO: 7 as a core sequence, (ii) IL-18 and functional equivalents thereof effectively inhibit osteoclast

formation, and (iii) the inhibition is mainly due to the action of GM-CSF induced and produced by IL-18.

Based on these, the present inventors solved the present object by an osteoclastgenic inhibitory agent comprising IL-18 or its functional equivalent as an effective ingredient.

Brief Description of the Accompanying Drawings

FIG. 1 shows the structure of the recombinant DNA pKGFHH2.

FIG. 2 shows the structure of the recombinant DNA pCSHIGIF/MUT35.

FIG. 3 shows the structure of the recombinant DNA pCSHIGIF/MUT42.

FIG. 4 shows the structure of the recombinant DNA pBGHuGF.

FIG. 5 shows the structure of the recombinant DNA pKGFMH2.

In these figures, KGFHH2 cDNA means a cDNA encoding the IL-18 according to the present invention: IGIF/MUT35; a DNA encoding the IL-18 according to the present invention: IGIF/MUT42; a DNA encoding the IL-18 according to the present invention: HuIGIF; a chromosomal DNA encoding the IL-18 according to the present invention: KGFMH2 cDNA; a cDNA encoding the IL-18 according to the present invention: 5S; a gene for 5S ribosomal RNA: Ptac; a tac promoter: rrnBT1T2; a termination region of a ribosomal RNA operon: AmpR; an ampicillin resistent gene: pBR322ori; a replication origin of

Escherichia coli: CMV; a cytomegalovirus promoter: IFNss; a nucleotide sequence encoding a signal peptide for subtype α2b of human interferon-α.

Detailed Description of the Invention

The present invention relates to an osteoclastgenic inhibitory agent comprising IL-18 or its functional equivalent as an effective ingredient. The wording "**IL-18**" as referred to in the invention includes polypeptides with the above property independently of their sources and origins. For example, the IL-18 used in the present invention includes, as internal partial amino acid sequences, the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3, as well as SEQ ID NO: 4 and SEQ ID NO: 5, and includes the amino acid sequence of SEQ ID NO: 6 or SEQ ID NO: 7 as a whole. The wording "**functional equivalent(s)**" as referred to in the present invention includes (i) those wherein one or more amino acids in the amino acid sequence of IL-18 are replaced with different amino acids, (ii) those wherein one or more amino acids are added to the N- and/or C-termini of the amino acid sequence of IL-18, (iii) those wherein one or more amino acids are inserted into the internal sites of the amino acid sequence of IL-18, (iv) those wherein one or more amino acids in the N- and/or C-terminal regions of the amino acid sequence of IL-18 are deleted, and (v) those wherein one or more amino acids in the internal regions of the amino acid sequence of IL-18 are deleted; all of these modifications should be made within the range that does not

substantially lose the property of osteoclast formation by IL-18 among the inherent property of IL-18. Examples of such functional equivalents are described along with their detailed amino acid sequences in Japanese Patent Application No. 20,906/97 by the same applicant of the present applicant, i.e., polypeptides which are capable of inducing production of interferon-gamma by immunocompetent cells, wherein said polypeptides contain either amino acid sequence wherein one or more cysteines are replaced with different amino acid(s) while leaving respective consensus sequences as shown in SEQ ID NOs: 1, 2 and 4 intact, or that wherein one or more amino acids are added, removed and/or replaced at one or more sites including those in the consensus sequences but excluding those of the replaced cysteine. The different amino acids to replace the cysteine(s) are not restricted to any types, as far as the resulting polypeptide, containing an amino acid sequence replaced with the different amino acid(s), exhibits an activity of inducing production of IFN-γ by immunocompetent cells in the presence or absence of an appropriate cofactor, as the wild-type polypeptides containing SEQ ID NOs: 1, 2 and 4 as consensus partial amino acid sequences, and a stability significantly higher than that of the wild-type polypeptides. The different amino acids include serine, threonine, alanine, valine, leucine, isoleucine, histidine, tyrosine, phenylalanine, tryptophan, and methionine, among which the most preferable amino acid is serine or alanine. Embodiments of the amino acid sequences, containing SEQ ID NOs: 1, 2 and 4 as consensus partial amino acid sequences, in which one or more cysteines are to be replaced

with different amino acid(s) are the wild-type polypeptides containing SEQ ID NO: 6 or 7. SEQ ID NO: 6 contains cysteines at the 38th, 68th, 76th, and 127th positions from the N-terminus. SEQ ID NO: 7 contains cysteines at the 7th, 75th, and 125th positions. The polypeptides include those containing the amino acid sequence of any one of SEQ ID NOs: 20-26, which are derived from the wild-type polypeptide containing SEQ ID NO: 6, those containing the amino acid sequence of SEQ ID NO: 27 or 28, which are derived from the wild-type polypeptide containing the amino acid sequence of SEQ ID NO: 7, and those containing an amino acid sequence derived from any one of SEQ ID NOs: 20-28 by adding, removing, and/or replacing one or more amino acids to and/or at position(s) excepting the positions where the cysteine(s) have been replaced while retaining the desired biological activities and stability. The wording "one or more amino acids" means the number of amino acids which conventional methods such as site-directed mutagenesis can usually add, remove or replace. The polypeptides containing any one of SEQ ID NOs: 20-28 possess both stability and biological activities significantly higher than those of the wild-type polypeptides.

The functional equivalents as referred to in the present invention further include glycosylated polypeptides of IL-18 and the above polypeptides. Any of these IL-18 and functional equivalents thereof, both of which are included to and referred to as "**IL-18**" in the present invention, unless specified otherwise, can be used in the present invention independently of their origins; those prepared by separating from natural sources such as cell cultures and from artificially

synthesized ones using recombinant DNA technology and peptide synthesis.

With economical viewpoint, methods of recombinant DNA technology are advantageously used; generally, desired IL-18 can be obtained by introducing DNAs encoding IL-18 into appropriate hosts derived from microorganisms, plants, and animals to form transformants, culturing the transformants in nutrient culture media in a conventional manner, and purifying the cultures by conventional methods used for purifying cytokines. Any DNAs can be used as the above DNAs as long as they contain a DNA encoding IL-18, and can be suitably selected depending on the purpose of the use of the present osteoclastgenic inhibitory agent or on the recombinant DNA technology used. For example, Japanese Patent Kokai Nos. 193,098/96, 231,598/96, and 27,189/96 by the same applicant of the present invention disclose in detail methods for producing IL-18 by culturing transformed microorganisms into which DNAs including a cDNA encoding mouse or human IL-18 are introduced; and Japanese Patent Application No. 185,305/96 by the same applicant of the present invention discloses in detail a method for producing IL-18 encoding human IL-18 by culturing transformed animal cells which have an introduced DNA that includes a chromosomal DNA encodes human IL-18. Japanese Patent Application No. 20,906/97 by the same applicant of the present invention discloses in detail a method for producing IL-18 by culturing transformed animal cells having an introduced DNA which includes a DNA encoding a functional equivalent of human IL-18.

The aforesaid recombinant DNA technology has an

economical advantage, but depending on the hosts and DNA sequences used, the IL-18 thus obtained may have somewhat different physicochemical property from those of IL-18 produced and functions *in vivo*. Japanese Patent Application No. 67,434/96 by the same applicant of the present invention discloses in detail a preparation of IL-18 using established human cell lines as natural sources, and Japanese Patent Application No. 213,267/96 by the same applicant also discloses in detail the preparation using an interleukin-1β-converting enzyme. The IL-18 obtained by those preparations can be estimated to have substantially the same or equal physicochemical property to that of IL-18 that is produced and functions *in vivo*, and the yield can be estimated to be slightly lower. However, such IL-18 has an advantage that it has a fewer side effects when used as pharmaceuticals directed to administering to warm-blooded animals in general and including humans. When applying purification methods using monoclonal antibodies specific to IL-18, as disclosed in Japanese Patent Application No. 231,598/96 by the same applicant of the present invention, a relatively-high purity IL-18 can be obtained in a minimum labor and cost.

The present osteoclastgenic inhibitory agent comprising the aforesaid IL-18 includes any types and forms usable to inhibit osteoclast formation both *in vivo* and *in vitro*. The present agent can be advantageously used as ingredients for cell culture media for animal cells, which satisfactorily inhibit osteoclast formation, maintain, proliferate, and/or differentiate the desired cells; components

of screening kits for bone-related therapeutic agents; bone-resorption regulatory agents; and agents for osteoclast-related diseases. The bone-resorption regulatory agents include medicaments and health foods that exert an osteoclastgenic inhibitory activity *in vivo*, control bone resorption to normal conditions, and improve unfavorable physical conditions such as a relatively-insignificant arthralgia. The agents for osteoclast-related diseases include medicaments used to prevent and/or treat diseases caused by an excessive osteoclast formation and/or its function. Examples of such diseases are hypercalcemia, osteoclastoma, Behçet's syndrome, osteosarcoma, arthropathy, chronic rheumatoid arthritis, deformity ostitis, primary hyperthyroidism, osteopenia, and osteoporosis. Varying depending on the types of agents and diseases to be treated, the present agent is usually formulated into a liquid, paste, or solid form which contains 0.000002-100 w/w %, preferably, 0.0002-0.5 w/w % of IL-18.

The present osteoclastgenic inhibitory agent can be IL-18 alone or compositions comprising IL-18 and one or more other ingredients such as carriers, excipients, diluents, adjuvants, antibiotics, and proteins such as serum albumin and gelatin as stabilizers; saccharides such as glucose, maltose, maltotriose, maltotetraose, trehalose, sucrose, isomaltose, lactose, panose, erlose, palatinose, lactosucrose, raffinose, fructooligosaccharide, galactooligosaccharide, lentinan, dextrin, pullulan, and sugar alcohols including sorbitol, maltitol, lactitol, and maltotriitol; buffers comprising phosphates or citrates mainly; and reductants such as 2-

mercaptoethanol, dithiothreitol, and reduced glutathione; and optionally biologically active substances such as interferon-α, interferon-β, interferon-γ, interleukin-2, interleukin-3, interleukin-6, interleukin-12, TNF-α, TNF-β, GM-CSF, estrogen, progesterone, chlormadinone acetate, calcitonin, somatokine, somatomedin, insulin-like growth factor, ipriflavone, parathyroid hormone (PTH), norethisterone, busulfan, ancitabine, cytarabine, fluorouracil, tetrahydrofurfuryl fluorouracil, methotrexate, vitamin D_2, active vitamin D, Krestin® or polysaccharide K, L-asparaginase, and OK-432 or Picibanil®; and calcium salts such as calcium lactate, calcium chloride, calcium monohydrogenphosphate, and L-calcium L-aspartate. When used as agents for administering to warm-blooded animals in general and including humans, i.e., agents for osteoclast-related diseases, the present agent can be preferably formulated into compositions by appropriately combining with one or more of the above physiologically-acceptable substances.

The present osteoclastgenic inhibitory agent includes medicaments in a unit dose form used for administering to warm-blooded animals in general and including humans. The wording "**unit dose form**" means those which contain IL-18 in an amount suitable for a daily dose or in an amount up to four fold by integers or up to 1/40 fold of the dose, and those in a physically separated and formulated form suitable for prescribed administrations. Examples of such formulations are injections, liquids, powders, granules, tablets, capsules, troches, collyriums, nebulas, and suppositories.

The present agent as an osteoclastgenic inhibitory

agent effectively treat and prevent osteoclast-related diseases independently of oral and parenteral administrations. Varying depending on the types and symptoms of patients' diseases, the present agent can be administered to the patients orally, intradermally, subcutaneously, muscularly, or intravenously at a dose of about 0.5 μg to 100 mg per shot, preferably, at a dose of about 2 μg to 10 mg per shot of IL-18, 2-6 fold a day or 2-10 fold a week for one day to one year.

In the below, with reference to experiments, the preparation, physicochemical property, and biological activity of the IL-18 according to the present invention are described:

Experiment 1

Preparation of human IL-18

According to the method in Japanese Patent Kokai No. 231,598/96 by the same applicant of the present invention, an autonomously-replicable recombinant DNA, **pKGFHH2**, linked to a cDNA encoding human IL-18, was prepared. Dideoxyribonucleotide sequencing analyzed that, as shown in FIG. 1, in the recombinant DNA, KGFHH2 cDNA containing the base sequence of SEQ ID NO: 8 was linked to the downstream of Ptac, a Tac promoter. The recombinant DNA pKGFHH2 contained the amino acid sequences of SEQ ID NOs: 1 to 5; these amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 8.

According to the method in Japanese Patent Kokai No. 231,598/96, the recombinant DNA pKGFHH2 was introduced into an *Escherichia coli* Y1090 strain, ATCC 37197, and the strain was cultured. The produced polypeptide was purified by

immunoaffinity chromatography to obtain a purified human IL-18 with a purity of at least 95% in a yield of about 25 mg/ℓ culture. According to the method in Japanese Patent Kokai No. 193,098/96 by the same applicant of the present invention, the purified human IL-18 was analyzed for biological activity and physicochemical property as indicated below: When culturing human lymphocytes, collected by a conventional manner from a healthy donor, in the presence of the purified human IL-18, IFN-γ production was observed depending on the concentration of IL-18, resulting in a confirmation that IL-18 has an activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified IL-18 was subjected to SDS-PAGE, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 18,500±3,000 daltons. The IL-18 gave a pI of 4.9±1.0 as determined by conventional chromatofocusing. Conventional analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the IL-18 had the amino acid sequence of SEQ ID NO: 9, i.e., the amino acid sequence of SEQ ID NO: 8 where a methionine residue was linked to the N-terminus.

Experiment 2

Preparation of human IL-18

According to the method in Japanese Patent Application No. 67,434/96 by the same applicant of the present invention, THP-1 cells, ATCC TIB 202, a human monocyte cell line derived from a male with acute monocytic leukemia, were inoculated to

the dorsum subcutaneous tissues of new born hamsters, followed by feeding the hamsters for three weeks. Tumor masses, about 15 g weight each, formed in the subcutaneous tissues of each hamster, were extracted, dispersed in media, and disrupted. The polypeptide obtained from the disrupted cells was purified by immunoaffinity chromatography to obtain a purified human IL-18 in a yield of an about 50 ng/head.

Similarly, according to the method in Japanese Patent Application No. 67,434/96, the purified human IL-18 was analyzed and determined for biological activity and physicochemical property as indicated below: It was confirmed that culturing human lymphocytes, collected from healthy donors in a conventional manner, in the presence of different concentrations of the human IL-18, resulted in an IL-18 dose-dependent IFN-γ production. This revealed that the human IL-18 has a biological activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE using 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ production inducing activity at a position corresponding to 18,000-19,500 daltons. According to the peptide map disclosed in Japanese Patent Application No. 67,434/96, the human IL-18 was treated with clostripain commercialized by Sigma Chemical Company, Missouri, USA, to obtain polypeptide fragments, followed by subjecting the fragments for fractionation to high-performance liquid chromatography (HPLC) using "**ODS-120T**", a column commercialized

by Tosoh Corporation, Tokyo, Japan, and analyzing the amino acid sequences of the fragments from the N-terminus to reveal the following amino acid sequences of SEQ ID NOs: 10 to 13. These amino acid sequences were completely coincided with amino acids 148-157, 1-13, 45-58, and 80-96 in SEQ ID NO: 6. The data shows that the human IL-18 obtained in Experiment 2 has the amino acid sequence of SEQ ID NO: 6 and all the partial amino acid sequences of SEQ ID NOs: 1 to 5.

Experiment 3

Preparation of functional equivalents

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, it was prepared an autonomously-replicable recombinant DNA, **pCSHIGIF/MUT35**, was linked to a DNA encoding a functional equivalent of human IL-18 where cysteines 38, 68, and 76 in SEQ ID NO: 6 were respectively replaced with serine, serine, and alanine. Dideoxyribonucleotide sequence analysis revealed that as shown in FIG. 2, in the recombinant DNA, DNA IGIF/MUT35 with SEQ ID NO: 14 linked to the downstream of a base sequence encoding a signal peptide of subtype α2b in human interferon-α in the same reading-frame, as reported by K. Henco et al., in *Journal of Molecular Biology*, Vol. 185, pp. 227-260 (1985), and had a stop codon for protein synthesis at further downstream. As shown in parallel in SEQ ID NO: 14, the amino acid sequence encoded by the recombinant DNA corresponded to SEQ ID NO: 6 where cysteines 38, 68, and 76 in SEQ ID NO: 6 were respectively replaced with serine, serine, and alanine. The recombinant DNA contained a nucleotide which encodes all the amino acid

sequences of SEQ ID NOs: 1 to 4 and the one of SEQ ID NO: 5 where cysteine at amino acid 5 in SEQ ID NO: 5 was replaced with alanine. These amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 14.

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, the recombinant DNA pCSHIGIF/MUT35 was introduced into COS-1 cells, ATCC CRL 1650, an established cell line derived from SV40 transformed African Green monkey kidney, followed by culturing the transformed cells. The produced polypeptide in the culture was purified by immunoaffinity chromatography to obtain a purified functional equivalent of human IL-18 in a yield of about 40 ng/ml culture. According to the method in Japanese Patent Application No. 20,906/97, the purified functional equivalent was analyzed and determined for biological activity and physicochemical property as indicated below: When culturing KG-1 cells, ATCC CCL 246, an established cell line derived from human acute myelogenous leukemia, in the presence of different concentrations of the purified functional equivalent of human IL-18, IFN-γ production was observed depending on the concentration of the IL-18, revealing that the IL-18 has a biological activity of inducing IFN-γ production by KG-1 cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified functional equivalent was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ production inducing

activity at a position corresponding to 18,000-19,500 daltons. Conventional analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the N-terminal region of the functional equivalent had the amino acid sequence of SEQ ID NO: 15 which corresponded to the amino acid sequence in the N-terminal region as shown in parallel in SEQ ID NO: 14.

Experiment 4

Preparation of functional equivalent

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, it was prepared an autonomously-replicable recombinant DNA, **pCSHIGIF/MUT42**, which was linked to a DNA encoding for a functional equivalent of human IL-18 where cysteines 38, 68, 76, and 127 in SEQ ID NO: 6 were respectively replaced with serine, serine, alanine, and serine. Dideoxyribonucleotide sequencing revealed that, as shown in FIG. 3, in the recombinant DNA, DNA IGIF/MUT42 with SEQ ID NO: 16 linked to the downstream of a base sequence encoding a signal peptide for subtype α2b of human interferon-α in the same reading frame, as reported by K. Henco et al., in *Journal of Molecular Biology*, Vol. 185, pp. 227-260 (1985), and had a stop codon for protein synthesis at further downstream. As shown in parallel in SEQ ID NO: 16, the amino acid sequence encoded by the recombinant DNA corresponded to SEQ ID NO: 6 where cysteines 38, 68, 76, and 127 in SEQ ID NO: 6 were respectively replaced with serine, serine, alanine, and serine. The recombinant DNA contained a nucleotide sequence which encodes all the amino acid sequences of SEQ ID NOs: 1 to

4 and the one of SEQ ID NO: 5 where cysteine 5 in SEQ ID NO: 5 was replaced with alanine. These amino acid sequences were respectively encoded by nucleotides 46-63, 88-105, 400-420, 151-165, and 214-228 in SEQ ID NO: 16.

According to the method in Japanese Patent Application No. 20,906/97 by the same applicant of the present invention, the recombinant DNA pCSHIGIF/MUT42 was introduced into COS-1 cells, followed by culturing the cells. The produced polypeptide in the culture was purified by immunoaffinity chromatography to obtain a purified functional equivalent of human IL-18 in a yield of about 20 ng/ml culture. According to the method in Japanese Patent Application No. 20,906/97, the purified functional equivalent was analyzed and determined for biological activity and physicochemical property as indicated below: When cultured KG-1 cells in the presence of different concentrations of the purified functional equivalent, a dose-dependent IFN-γ production was observed, and this revealed that the functional equivalent has a biological activity of inducing IFN-γ production by KG-1 cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified functional equivalent was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 18,000-19,500 daltons. Conventional analysis using "**PROTEIN SEQUENCER MODEL 473A**", an apparatus of Applied Biosystems, Inc., Foster City, USA, revealed that the N-terminal region of the functional equivalent had the amino acid sequence of SEQ ID NO:

15 which completely corresponded to the amino acid sequence in the N-terminal region as shown in parallel in SEQ ID NO: 16.

Experiment 5

Preparation of human IL-18

According to the method in Japanese Patent Application No. 185,305/96 by the same applicant of the present invention, an autonomously-replicable recombinant DNA, **pBGHuGF**, linked to a chromosomal DNA encoding human IL-18, was obtained. Dideoxyribonucleotide sequencing analysis revealed that as shown in FIG. 4, in the recombinant DNA, a chromosomal DNA, which encodes human IL-18, i.e., DNA HuIGIF with SEQ ID NO: 17, was linked to the downstream of a restriction site by a restriction enzyme, *Hind* III. As shown in SEQ ID NO: 17, the chromosomal DNA HuIGIF consists of 11,464 bp where the exon was fragmented by four introns positioning at nucleotides 83-1,453, 1,466-4,848, 4,984-6,317, and 6,452-11,224. Among the resting nucleotide sequence excluding these introns, nucleotides 3-11,443 from the 5'-terminus are the part that encodes a precursor of human IL-18, and nucleotides 4,866-4,983 are the part that encodes an active human IL-18. The chromosomal DNA contained nucleotides sequences encoding SEQ ID NOs: 1 to 5; these amino acid sequences were respectively encoded by nucleotides 4,911-4,928, 4,953-4,970, 11,372-11,392, 6,350-6,364, and 6,413-6,427 in SEQ ID NO: 17.

According to the method in Japanese Patent Application No. 185,305/96, the recombinant DNA pBGHuGF was introduced into CHO-K1 cells, ATCC CCL 61, an established cell line derived from Chinese hamster ovary, followed by culturing the cells. The

culture supernatant was contacted with a supernatant of cell disruptant prepared from a THP-1 cell culture to produce a polypeptide which was then purified by immunoaffinity chromatography to obtain a purified human IL-18 in a yield of about 15 mg/ℓ culture. According to the method in Japanese Patent Application No. 185,305/96, the polypeptide was analyzed and determined for biological activity and physicochemical property as indicated below: It was confirmed that human lymphocytes, which were collected from a healthy donor, produced IFN-γ depending on the purified human IL-18 concentration when cultured at different concentrations of the human IL-18, revealing that the human IL-18 has a biological activity of inducing IFN-γ production by lymphocytes as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE in the presence of 2 w/v % dithiothreitol as a reductant, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 18,000-19,500 daltons. The N-terminal region of the human IL-18 contained the amino acid sequence of SEQ ID NO: 15 which completely corresponded to the amino acid sequence in the N-terminal region of SEQ ID NO: 17 for an active IL-18.

Experiment 6

Preparation of mouse IL-18

To a 0.5-ml reaction tube were added 8 µl of 25 mM magnesium chloride, 10 µl of 10 x PCR buffer, one µl of 25 mM dNTP mix, one µl of 2.5 units/µl of amplitaq DNA polymerase, one ng of a recombinant DNA, which encodes mouse IL-18 having the

nucleotide sequence of SEQ ID NO: 18 and the amino acid sequence of SEQ ID NO: 7, prepared from a phage DNA clone according to the method in Japanese Patent Kokai No. 27,189/96, and adequate amounts of a sense and antisense primers having nucleotide sequences represented by 5'-ATAGAATTCAAATGAACTTTGGCCGACTTCACTG-3' and 5'-ATAAAGCTTCTAACTTTGATGTAAGTT-3', respectively, which were chemically synthesized based on the amino acid sequences nearness to the N- and C-termini of SEQ ID NO: 7, and the mixture solution was brought up to a volume of 100 µl with sterilized distilled water. The solution thus obtained was subjected in a usual manner to PCR reaction of the following three cycles of successive incubations at 94°C for one minute, 43°C for one minute, and 72°C for one minute, and further 40 cycles of successive incubations at 94°C for one minute, 60°C for one minute, and 72°C for one minute.

The product obtained by the PCR reaction and "**pCR-Script SK** (+)", a plasmid vector commercialized by Stratagene Cloning Systems, California, USA, were in a conventional manner ligated together using a DNA ligase into a recombinant DNA which was then introduced into "**XL-1 Blue MRF'Kan**", an *Escherichia coli* strain commercialized by Stratagene Cloning Systems, California, USA., to obtain a transformant. The transformant was inoculated to L-broth (pH 7.2) containing 50 µg/ml ampicillin, followed by the incubation at 37°C for 18 hours under shaking conditions. The culture was centrifuged to obtain the proliferated transformants which were then treated with a conventional alkali-SDS method to isolate a recombinant DNA. A portion of the recombinant DNA isolated was analyzed by

dideoxyribonucleotide sequencing, revealing that the recombinant DNA contained restriction sites of *Eco* RI and *Hind* III at the 5'- and 3'-termini of SEQ ID NO: 18, respectively, and a DNA containing a methionine codon for initiating polypeptide synthesis and a TAG codon for terminating polypeptide synthesis, which were located in just before and after the N- and C-termini of the amino acid sequence as shown in parallel in SEQ ID NO: 18. The recombinant DNA contained the nucleotide sequences of SEQ ID NOs: 1 to 5. These amino acid sequences were encoded by nucleotides 46-63, 85-102, 394-414, 148-162, and 211-225 in SEQ ID NO: 18.

The remaining portion of the recombinant DNA was in a conventional manner cleaved with restriction enzymes of *Eco* RI and *Hind* II, and the resulting 0.1 μg of an *Eco* RI-*Hind* III DNA fragments, obtained by using "**DNA LIGATION KIT VER 2**", a DNA ligation kit commercialized by Takara Shuzo Co., Ltd., Tokyo, Japan, and 10 ng of pKK223-3, a plasmid vector commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been cleaved with a restriction enzyme were linked together, by incubating at 16°C for 30 min to obtain an autonomously-replicable recombinant DNA, **pKGFMH2.** Using competent cell method, an *Escherichia coli* Y1090 strain, ATCC 37197, was transformed using the recombinant DNA pKGFMH2, and the resulting transformant, KGFMH2, was inoculated to L-broth (pH 7.2) containing 50 μg/ml ampicillin, and cultured at 37°C for 18 hours under shaking conditions. The culture was centrifuged to collect the proliferated transformants, followed by applying a conventional SDS-alkali method to a portion of the transformants

to extract the recombinant DNA pKGFMH2. Dideoxyribonucleotide sequencing analysis revealed that, as shown in FIG. 5, KGFMH2 cDNA containing the nucleotide sequence of SEQ ID NO: 18 was linked to the downstream of the Tac promoter in the recombinant DNA pKGFMH2.

Ampicillin was added to L-broth (pH 7.2), which had been sterilized by autoclaving, to give a concentration of 50 μg/ml, cooled to 37°C, and inoculated with the transformant KGFMH2, followed by the culture at 37°C for 18 hours. Eighteen liters of a fresh preparation of the same culture medium was placed in a 20-ℓ jar fermenter, similarly sterilized as above, admixed with ampicillin, cooled to 37°C, and inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 37°C for 8 hours under aeration-agitation conditions. The resulting culture was centrifuged to collect the cultured cells which were then suspended in a mixture solution (pH 7.3) containing 150 mM sodium chloride, 16 mM disodium hydrogenphosphate, and 4 mM sodium dihydrogenphosphate, disrupted by ultrasonication, and centrifuged to remove cell disruptant, and this yielded an about two liters of a supernatant.

To an about two liters of the supernatant was added 10 mM phosphate buffer (pH 7.3) containing ammonium sulfate to give a 40% ammonium saturation. The resulting sediment was removed by centrifugation, and the supernatant was mixed with ammonium sulfate to give an 85% ammonium saturation, allowed to stand at 4°C for 18 hours, and centrifuged at about 8,000 rpm for 30 min to obtain a newly formed sediment. The sediment thus

obtained was dissolved in 10 mM phosphate buffer (pH 6.6) containing 1.5 M ammonium sulfate to give a total volume of about 1,300 ml, and the solution was filtered, and fed to a column packed with about 800 ml of "**PHENYL SEPHAROSE CL-6B**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, followed by washing the column with a fresh preparation of the same buffer and feeding to the column a linear gradient buffer of ammonium sulfate decreasing from 1.5 M to 0 M in 10 mM phosphate buffer (pH 6.6) at an SV (space velocity) 1.5. Fractions eluted at around 1 M ammonium sulfate were pooled, concentrated using a membrane filter, and dialyzed against 10 mM phosphate buffer (pH 6.5) at 4°C for 18 hours. The dialyzed solution was fed to a column packed with about 55 ml of "**DEAE-5PW**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been equilibrated with 10 mM phosphate buffer (pH 6.5). The column was washed with a fresh preparation of the same buffer, and fed with a linear gradient buffer of sodium chloride increasing from 0 M to 0.5 M in 10 mM phosphate buffer (pH 6.5) at SV 5.5, followed by collecting fractions eluted at around 0.2 M sodium chloride. Thereafter, the fractions were pooled and concentrated similarly as above up to give an about nine milliliters, followed by dialyzing the concentrate against PBS (phosphate buffered saline) at 4°C for 18 hours, and feeding the dialyzed solution to a column packed with "**SUPERDEX 75**", a gel commercialized by Pharmacia LKB Biotechnology AB, Uppsala, Sweden, which had been equilibrated with a fresh preparation of the same PBS. The column was fed with a fresh preparation of the same PBS to collect fractions

with an IFN-γ inducing activity, and the fractions were pooled and concentrated with a membrane filter to obtain a purified mouse IL-18 in a yield of about 350 μg/ℓ culture.

According to the method in Japanese Patent Kokai No. 27,189/96, the purified mouse IL-18 was analyzed and determined for biological activity and physicochemical property as indicated below: Culturing mouse spleen cells, collected by a conventional manner, under different concentrations of the mouse IL-18 resulted in an IFN-γ production depending on the concentrations of the mouse IL-18, and this revealed that the mouse IL-18 has an activity of inducing IFN-γ production by spleen cells as an immunocompetent cell. In accordance with the method as reported by U. K. Laemmli in *Nature*, Vol. 227, pp. 680-685 (1970), the purified human IL-18 was subjected to SDS-PAGE under non-reducing conditions, resulting in a major band with an IFN-γ inducing activity at a position corresponding to 19,000±5,000 daltons. The N-terminal region of the mouse IL-18 contained the amino acid sequence of SEQ ID NO: 19 which corresponded to the N-terminal region of SEQ ID NO: 18.

With reference to Experiment 7, the biological activity of the IL-18 according to the present invention will be described in more detail, and Experiment 8 describes the cytotoxicity of the IL-18:

Experiment 7

Biological activity

Experiment 7-1

Induction of GM-CSF production

Using a heparinized syringe, blood was collected from

a healthy volunteer and diluted two fold with serum-free RPMI 1640 medium (pH 7.4). The diluent was overlaid on a ficoll and centrifuged, and the collected lymphocytes were washed with RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum, and suspended in a fresh preparation of the same medium to give a cell density of 1 x 10^6 cells/ml, followed by distributing the cell suspension to a 12-well microplate by two ml/well.

Using RPMI 1640 medium (pH 7.4) supplemented with 10 v/v % fetal calf serum, an IL-18 preparation obtained by the method in Experiment 1 was prepared into a one µg/ml solution which was then distributed to the above microplate by 20-200 µl/well. To the microplate was further added a fresh preparation of the same buffer, supplemented with 500 µl/ml of Concanavalin A, by 10 µl/well, followed by the incubation at 37°C for 48 hours in a 5 v/v % CO_2 incubator. After completion of the culture, supernatants in each well were sampled by 0.1 ml/well, and determined for GM-CSF content using a conventional enzyme immunoassay. In parallel, a culture system free of IL-18 as a control was provided and treated similarly as above. The data is in Table 1:

Table 1

IL-18* (nM)	GM-CSF yield (pg/ml)
0	510
0.7	2,150
2.8	3,050
5.6	3,950

Note: The symbol "*" means that IL-18 was added to the culture system in the presence of 2.5 μg/ml of Concanavalin A.

The results in Table 1 indicate that lymphocytes as an immunocompetent cell produced GM-CSF depending on the concentration of IL-18 when contacted with IL-18 in the presence of Concanavalin A as a cofactor. It was also confirmed that all of the IL-18 preparations and functional equivalents thereof, which were obtained by the methods in Experiments 2 to 5, induced GM-CSF production even when used alone similarly as above. An IL-18 preparation obtained by the method in Experiment 6 was tested in accordance with Experiment 7-1 except that the human lymphocytes used in the experiment were replaced with spleen cells prepared from mouse by a conventional manner, revealing that the IL-18 preparation also induced GM-CSF production.

Experiment 7-2

Inhibition of osteoclast formation

Experiment 7-2(a)

As reported by T. J. Martin and K. W. Ng in *Journal of Cellular Biochemistry*, Vol. 56, pp. 357-366 (1994), it is considered requisite for contacting osteoclastic precursor cells, derived from hematopoietic stem cells, with osteoblasts or bone marrow stromas to generally differentiate osteoclastic precursor cells into mature osteoclasts. As described by G. D. Roodman in *Endocrine Reviews*, Vol. 17, No. 4, pp. 308-332 (1996), it is generally recognized that osteoclasts have characters of multinucleated cells, tartaric acid-resistant acid

phosphatase (hereinafter abbreviated as "**TRAP**") activity, and a calcitonin receptor. In a co-culture system of osteoblasts and bone marrow cells as reported by Nobuyuki UDAGAWA et al., in *Journal of Experimental Medicine*, Vol. 182, pp. 1,461-1,468 (1995), these cells respond to factors such as 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, adrenocortical hormone, interleukin 1, interleukin 6, and interleukin 11, to form osteoclast-like cells (hereinafter may be abbreviated as "**OCL**"). The formed OCL has characters of osteoclasts *in vivo*. Therefore, the co-culture system well reflects *in vitro* the processes of osteoclast formation *in vivo*. Using this system, experiments for osteoclast formation and osteoclastgenic inhibitory agents can be carried out.

The osteoclastgenic inhibitory activity of the IL-18 according to the present invention was studied using the above co-culture system. The osteoblasts used in this experiment were prepared in a conventional manner by treating a newborn mouse calvaria with 0.1 w/v % collagenase commercialized by Worthington Biochemical Co., Freefold, Australia, and 0.2 w/v % dispase commercialized by Godo Shusei Co., Ltd., Tokyo, Japan. The bone marrow cells were prepared from a mature mouse in a conventional manner. As a negative control, 2×10^4 cells of a primary cell culture of osteoblasts and 5×10^5 cells of bone marrow cells were co-cultured in each well of a 48-well microplate containing 0.4 ml/well of α-MEM medium supplemented with 10 v/v % fetal calf serum (hereinafter designated as "**Medium**" throughout Experiment 4-2) at 37°C for seven days in a 5 v/v % CO_2 incubator. As a positive control, the above two-

types of cells were co-cultured similarly as in the negative control except that they were cultured in other wells containing 10^{-8}M of 1α,25-dihydroxyvitamin D_3 commercialized by Wako Pure Chemicals, Tokyo, Japan, and 10^{-7}M of prostaglandin E_2 commercialized by Sigma Chemical Company, Missouri, USA. The aforesaid two-types of cells were co-cultured similarly as in the positive control except that they were cultured in other wells containing 1α,25-dihydroxyvitamin D_3 commercialized by Wako Pure Chemicals, Tokyo, Japan, and prostaglandin E_2 commercialized by Sigma Chemical Company, Missouri, USA., in the same concentrations as used in the positive control, and a concentration of 0.01-10 ng/ml of an IL-18 preparation prepared by the method in Experiment 6. In every co-culture system, the media in each well were replaced with fresh preparations of the same media used in the co-culture systems on the 3rd day after the initiation of each culture. According to the method by Nobuyuki UDAGAWA in *Journal of Experimental Medicine*, Vol. 182, pp. 1,461-1,468 (1995), the cells on the 6th day after the initiation of each culture were fixed and stained based on TRAP activity, followed by counting the stained cells (hereinafter called "**TRAP-positive cells**") per well. Throughout Experiment 4-2, quadruplet wells under the same conditions were provided for each co-culture system, and the mean value for the TRAP-positive cells per well in each system was calculated. The results are in Table 2:

Table 2

IL-18 (ng/ml)	Osteoclastgenic formation factor*1	Number of TRAP-positive cells per well*2
0	-	2
0	+	110
0.01	+	114
0.1	+	111
0.5	+	106
1	+	63
2	+	29
4	+	12
8	+	2
10	+	2

Note: *1: The symbols of "+" and "-" show co-culture systems with and without 10^{-8}M 1α,25-dihydroxyvitamin D_3 and 10^{-7}M prostaglandin E_2, respectively.

*2: It shows a mean value of the data from quadruplet wells cultured under the same conditions.

As shown in Table 2, the formation of TRAP-positive cells was not substantially observed in the negative control, but the distinct formation was observed in the positive control. In the co-culture systems, i.e., the positive control supplemented additionally with IL-18, the formation of TRAP-positive cells was inhibited depending on the concentration of IL-18, and the maximum inhibition, i.e., a level equal to that in the negative control, was found at eight ng/ml or more of IL-18. These data strongly indicates that IL-18 has a concrete activity of inhibiting OCL formation *in vitro* and also inhibits osteoclast formation.

Experiment 7-2(b)

As described hereinbefore, it was confirmed that there exist factors that induce the formation of osteoclast-like cells in the co-culture systems used throughout Experiment 7-2. Therefore, in this Experiment 7-2(b), it was studied whether the inhibitory activity of IL-18 on osteoclast formation observed in Experiment 7-2(a) was specific to some factors or not; the osteoclast-like cells were cultured by the same method as used in the negative control in Experiment 7-2(a) except for using a medium supplemented with 10^{-8}M 1α,25-dihydroxyvitamin D_3, 10^{-7}M prostaglandin E_2, 200 ng/ml parathyroid hormone, 100 ng/ml interleukin 1, or 20 ng/ml interleukin 11. These culture systems were for positive controls. In parallel, the cells were cultured in other wells by the same method used in the positive controls except for using a medium containing 10 ng/ml of an IL-18 preparation obtained by the method in Experiment 6, in addition to any one of the above factors at the same

concentration. After completion of the cultures, TRAP-positive cells in each well were counted, and the numbers were compared similarly as in Experiment 7-2(a). The results are in Table 3:

Table 3

Osteoclast formation factor*1 (concentration)		IL-18*2	Number of TRAP-positive cells per well*3
D_3	(10^{-8}M)	-	94
		+	3
PGE_2	(10^{-7}M)	-	77
		+	3
PTH	(200 ng/ml)	-	63
		+	3
IL-11	(100 ng/ml)	-	84
		+	3
IL-1	(20 ng/ml)	-	71
		+	3

Note: *1: D_3, PGE_2, PTH, IL-11, and IL-1 are respectively 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, parathyroid hormone, interleukin-11, and interleukin-1 which were added to wells to give the concentrations as indicated in parentheses.

*2: The symbol "+" means that IL-18 was added to a well to give a concentration of 10 ng/ml, and the symbol "-" means that IL-18 was not added to.

*3: It shows a mean value of the data from quadruplet wells cultured under the same conditions.

As shown in Table 3, a distinct formation of TRAP-positive cells was observed in every positive control, but the formation was almost completely inhibited in the presence of IL-18. This strongly indicates that IL-18 has a wide and general activity of inhibiting osteoclast formation independently of osteoclast-formation-related factors.

Experiment 7-2(c)

It was studied whether the osteoclastgenic inhibition by IL-18, confirmed in Experiments 7-2(a) and 7-2(b), was caused by the action of the IL-18-induced GM-CSF. For positive and negative controls, the same co-culture systems employed in Experiment 7-2(a) were used. Using other wells, the co-culture of osteoblasts and bone marrow cells was carried out similarly as the method used for the positive controls except for using a medium supplemented with 1α,25-dihydroxyvitamin D_3 and prostaglandin E_2 at the same concentrations used in the positive control, and with (i) 10 µg/ml of an anti-mouse GM-CSF polyclonal antibody commercialized by R&D Systems, Minnesota, USA, (ii) 10 ng/ml of an IL-18 preparation obtained by the method in Experiment 6, (iii) (ii) plus 10 µg/ml of an anti-mouse polyclonal antibody, (iv) 0.1 ng/ml of a mouse GM-CSF commercialized by R&D Systems, Minnesota, USA, or (v) (iv) plus 10 µg/ml of an anti-mouse GM-CSF polyclonal antibody. After completion of the culture, TRAP-positive cells in each well were counted, and the numbers were compared similarly as in Experiment 7-2(a). The data is shown in Table 4 where the symbols "i" to "v" coincide with those used in the co-culture systems other than the control systems.

Table 4

Culture system*1	Osteoclastgenic factor*2	IL-18*3	GM-CSF*4	Anti-GM-CSF antibody*5	Number of TRAP-positive cells per well*6
N	-	-	-	-	3
P	+	-	-	-	122
i	+	-	-	+	112
ii	+	+	-	-	3
iii	+	+	-	+	111
iv	+	-	+	-	4
v	+	-	+	+	106

Note: *1; where the symbols "N" and "P" mean negative and positive controls, respectively, and the symbols "i" to "v" correspond to those in the five types co-culture systems used.

*2; where the symbol "+" means that 1α,25-dihydroxyvitamin D_3 and prostaglandin E_2 were respectively added to a well to give respective concentrations of 10^{-8}M and 10^{-7}M, and the symbol "-" means that these compounds were not added to.

*3; The symbol "+" means that IL-18 was added to a well to give a concentration of 10 ng/ml, and the symbol "-" means that IL-18 was not added to.

*4; The symbol "+" means that GM-CSF was added to a well to give a concentration of 0.1 ng/ml, and the symbol "-" means that GM-CSF was not added to.

*5; The symbol "+" means that an anti-GM-CSF polyclonal antibody was added to a well to give a concentration of 10 μg/ml, and the symbol "-" means that the polyclonal antibody was not added to.

As shown in Table 4, the formation of TRAP-positive cells was almost completely inhibited by IL-18, cf., the co-culture system (ii), but the inhibition was almost completely inhibited by the addition of the anti-mouse polyclonal antibody, cf., the co-culture system (iii). Mouse GM-CSF exhibited an activity of inhibiting the formation of TRAP-positive cells similar to IL-18, cf., the co-culture system (iv), and the inhibition was almost completely inhibited by the addition of the anti-mouse GM-CSF polyclonal antibody, cf., the co-culture system (v). The sole use of the anti-mouse GM-CSF polyclonal antibody gave no influence on the formation of TRAP-positive cells, cf., the co-culture system (i). These data strongly indicates that the osteoclastgenic inhibition by IL-18 was due to the action of the IL-18-induced GM-CSF.

Experiment 8

Acute toxicity test

Eight-week-old mice were in a conventional manner injected percutaneously, orally, or intraperitoneally with either of IL-18 preparations obtained by the methods in Experiments 1 to 6. The results showed that these IL-18 preparations had an LD_{50} of about one mg/kg or more in mice independent of the route of administration. The data evidences that IL-18 can be incorporated into pharmaceuticals for warm-blooded animals in general and including humans without causing no serious side effects.

As described in *Nikkei Biotechnology Annual Report 1996*, pp. 498-499 (1995), published by Nikkei BP Publisher, Tokyo, Japan (1995), the IL-18-induced GM-CSF has not yet been

clinically used in Japan, but applied clinically in USA and Europe. The fact would show that IL-18 has substantially no serious side effects. These facts indicate that the osteoclastgenic inhibitory agent according to the present invention can be successively administered to warm-blooded animals in general and including humans to induce osteoclast formation and exert a satisfactory therapeutic and/or prophylactic effect on osteoclast-related diseases without causing serious side effects.

The following Examples describe the present osteoclastgenic inhibitory agent according to the present invention:

Example 1

Liquid

Either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in physiological saline containing one w/v % human serum albumin as a stabilizer to give a concentration of two mg/ml of the IL-18 preparation. The resulting solutions were in a conventional manner membrane filtered for sterilization into liquids.

The liquids have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of an injection, ophthalmic solution, or collunarium for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 2

Dry agent

Fifty milligrams of either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in 100 ml of physiological saline containing one w/v % purified gelatin as a stabilizer. The solutions thus obtained were in a conventional manner membrane filtered for sterilization, distributed to vials by one milliliter, lyophilized, and sealed with caps.

The products have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of a dry injection for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 3

Dry agent

Fifty milligrams of either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, was dissolved in 100 ml of physiological saline containing one w/v % trehalose as a stabilizer. The solutions were in a conventional manner membrane filtered for sterilization, distributed to vials by one milliliter, lyophilized, and sealed with caps.

The products have a satisfactory stability and can be arbitrarily used as ingredients for cell culture and agents in the form of a dry injection for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 4

Ointment

"**HIVIS WAKO GEL® 104**", a carboxyvinylpolymer

commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, and a high-purity trehalose were dissolved in a sterilized distilled water to give respective concentrations of 1.4 w/w % and 2.0 w/w %, and the solution was mixed to homogeneity with either of IL-18 preparations obtained by the methods in Experiments 1 to 6, and adjusted to pH 7.2 to obtain a paste containing about one mg of an IL-18 preparation per g of the product.

Each product thus obtained has a satisfactory spreadability and stability and can be arbitrarily used as an agent in the form of an ointment for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Example 5

Tablet

"**FINETOSE**®", an anhydrous crystalline α-maltose powder commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, was mixed to homogeneity with either of IL-18 preparations, obtained by the methods in Experiments 1 to 6, and "**LUMIN**" or 1-1'-1"-trihepthyl-11-chinolyl(4)•4•4'-penthamethinchynocyanine-1,1"-dijodide. The mixtures were in a conventional manner tabletted to obtain tablets, about 200 mg weight each, containing an about two milligrams of either of the IL-18 preparations and an about two milligrams of LUMIN per tablet.

The products have a satisfactory swallowability, stability, and cell-activating activity and can be arbitrarily used as agents in the form of a tablet for regulating bone

resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

As described above, the osteoclastgenic inhibitory agent according to the present invention effectively inhibits osteoclast formation. Therefore, the agent can be arbitrarily used as an ingredient for cell culture and agents for regulating bone resorption and for osteoclast-related diseases, directed to treat and/or prevent hypercalcemia, osteoclastoma, osteoporosis, etc.

Thus the present invention with these useful activities and functions is a significant invention that would greatly contribute to this field.

While there has been described what is at present considered to be the preferred embodiments of the invention, it will be understood the various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirits and scope of the invention.

SEQUENCE LISTING

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patent In Release #1.0, Version #1.30

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Asn Asp Gln Val Leu Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Phe Glu Asp Met Thr Asp
1               5
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Phe Lys Leu Ile Leu Lys Lys
1               5
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Tyr Lys Asp Ser
1               5
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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Ser Thr Leu Ser Cys
1               5
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
```

```
    50                  55                  60
Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1               5                   10                  15
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
    50                  55                  60
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
        115                 120                 125
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human
(G) CELL TYPE: liver

(ix) FEATURE:
(A) NAME/KEY: mat peptide
(B) LOCATION: 1..471
(C) IDENTIFICATION METHOD: E

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT     48
```

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT      96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
ATG ACT GAT TCT GAC TGT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT     144
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC     192
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
TCT GTG AAG TGT GAG AAA ATT TCA ACT CTC TCC TGT GAG AAC AAA ATT     240
Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA     288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG     336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
ATG CAA TTT GAA TCT TCA TCA TAC GAA GGA TAC TTT CTA GCT TGT GAA     384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG     432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC                 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
Met Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Ser Ile Met Phe Thr Val Gln Asn Glu Asp
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Thr Ile Phe Ile Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
1               5                   10                  15
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: mat peptide
(B) LOCATION: 1..471
(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT      48
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                   10                  15
GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT      96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
```

```
ATG ACT GAT TCT GAC TCT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT     144
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
         35                  40                  45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC     192
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
     50                  55                  60
TCT GTG AAG TCT GAG AAA ATT TCA ACT CTC TCC GCT GAG AAC AAA ATT     240
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65                  70                  75                  80
ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA     288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                 85                  90                  95
AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG     336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
ATG CAA TTT GAA TCT TCA TCA TAC GAA GGA TAC TTT CTA GCT TGT GAA     384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG     432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC                 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: mat peptide
(B) LOCATION: 1..471
(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
TAC TTT GGC AAG CTT GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT      48
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                   10                  15
GAC CAA GTT CTC TTC ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT      96
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
ATG ACT GAT TCT GAC TCT AGA GAT AAT GCA CCC CGG ACC ATA TTT ATT     144
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC     192
```

```
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
TCT GTG AAG TCT GAG AAA ATT TCA ACT CTC TCC GCT GAG AAC AAA ATT     240
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65                  70                  75                  80
ATT TCC TTT AAG GAA ATG AAT CCT CCT GAT AAC ATC AAG GAT ACA AAA     288
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
AGT GAC ATC ATA TTC TTT CAG AGA AGT GTC CCA GGA CAT GAT AAT AAG     336
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
ATG CAA TTT GAA TCT TCA TCA TAC GAA GGA TAC TTT CTA GCT TCT GAA     384
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
        115                 120                 125
AAA GAG AGA GAC CTT TTT AAA CTC ATT TTG AAA AAA GAG GAT GAA TTG     432
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
GGG GAT AGA TCT ATA ATG TTC ACT GTT CAA AAC GAA GAC                 471
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 11464 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: human
- (G) CELL TYPE: placenta

(ix) FEATURE:
- (A) NAME/KEY: 5■ UTR
- (B) LOCATION: 1..3
- (C) IDENTIFICATION METHOD: E
- (A) NAME/KEY: leader peptide
- (B) LOCATION: 4..82
- (C) IDENTIFICATION METHOD: S
- (A) NAME/KEY: intron
- (B) LOCATION: 83..1453
- (C) IDENTIFICATION METHOD: E
- (A) NAME/KEY: leader peptide
- (B) LOCATION: 1454..1465
- (C) IDENTIFICATION METHOD: S
- (A) NAME/KEY: intron
- (B) LOCATION: 1466..4848
- (C) IDENTIFICATION METHOD: E
- (A) NAME/KEY: leader peptide
- (B) LOCATION: 4849..4865
- (C) IDENTIFICATION METHOD: S
- (A) NAME/KEY: mat peptide
- (B) LOCATION: 4866..4983
- (C) IDENTIFICATION METHOD: S
- (A) NAME/KEY: intron
- (B) LOCATION: 4984..6317
- (C) IDENTIFICATION METHOD: E
- (A) NAME/KEY: mat peptide
- (B) LOCATION: 6318..6451
- (C) IDENTIFICATION METHOD: S
- (A) NAME/KEY: intron
- (B) LOCATION: 6452..11224
- (C) IDENTIFICATION METHOD: E
- (A) NAME/KEY: mat peptide
- (B) LOCATION: 11225..11443

(C) IDENTIFICATION METHOD: S
(A) NAME/KEY: 3■ UTR
(B) LOCATION: 11444..11464
(C) IDENTIFICATION METHOD: E

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
AAG ATG GCT GCT GAA CCA GTA GAA GAC AAT TGC ATC AAC TTT GTG GCA        48
    Met Ala Ala Glu Pro Val Glu Asp Asn Cys Ile Asn Phe Val Ala
        -35                 -30                 -25
ATG AAA TTT ATT GAC AAT ACG CTT TAC TTT ATA G   GTAAGG CTAATGCCAT      98
Met Lys Phe Ile Asp Asn Thr Leu Tyr Phe Ile Ala
    -20             -15             -10
AGAACAAATA CCAGGTTCAG ATAAATCTAT TCAATTAGAA AAGATGTTGT GAGGTGAACT     158
ATTAAGTGAC TCTTTGTGTC ACCAAATTTC ACTGTAATAT TAATGGCTCT TAAAAAAATA     218
GTGGACCTCT AGAAATTAAC CACAACATGT CCAAGGTCTC AGCACCTTGT CACACCACGT     278
GTCCTGGCAC TTTAATCAGC AGTAGCTCAC TCTCCAGTTG GCAGTAAGTG CACATCATGA     338
AAATCCCAGT TTTCATGGGA AAATCCCAGT TTTCATTGGA TTTCCATGGG AAAAATCCCA     398
GTACAAAACT GGGTGCATTC AGGAAATACA ATTTCCCAAA GCAAATTGGC AAATTATGTA     458
AGAGATTCTC TAAATTTAGA GTTCCGTGAA TTACACCATT TTATGTAAAT ATGTTTGACA     518
AGTAAAAATT GATTCTTTTT TTTTTTTTCT GTTGCCCAGG CTGGAGTGCA GTGGCACAAT     578
CTCTGCTCAC TGCAACCTCC ACCTCCTGGG TTCAAGCAAT TCTCCTGCCT CAGCCTTCTG     638
AGTAGCTGGG ACTACAGGTG CATCCCGCCA TGCCTGGCTA ATTTTTGGGT ATTTTTACTA     698
GAGACAGGGT TTTGGCATGT TGTCCAGGCT GGTCTTGGAC TCCTGATCTC AGATGATCCT     758
CCTGGCTCGG GCTCCCAAAG TGCTGGGATT ACAGGCATGA ACCACCACAC ATGGCCTAAA     818
AATTGATTCT TATGATTAAT CTCCTGTGAA CAATTTGGCT TCATTTGAAA GTTTGCCTTC     878
ATTTGAAACC TTCATTTAAA AGCCTGAGCA ACAAAGTGAG ACCCCATCTC TACAAAAAAC     938
TGCAAAATAT CCTGTGGACA CCTCCTACCT TCTGTGGAGG CTGAAGCAGG AGGATCACTT     998
GAGCCTAGGA ATTTGAGCCT GCAGTGAGCT ATGATCCCAC CCCTACACTC CAGCCTGCAT    1058
GACAGTAGAC CCTGACACAC ACACACAAAA AAAAACCTTC ATAAAAAATT ATTAGTTGAT    1118
TTTTCTTAGG TGACTTTCCG TTTAAGCAAT AAATTTAAAA GTAAAATCTC TAATTTTAGA    1178
AAATTTATTT TTAGTTACAT ATTGAAATTT TTAAACCCTA GGTTTAAGTT TTATGTCTAA    1238
ATTACCTGAG AACACACTAA GTCTGATAAG CTTCATTTTA TGGGCCTTTT GGATGATTAT    1298
ATAATATTCT GATGAAAGCC AAGACAGACC CTTAAACCAT AAAAATAGGA GTTCGAGAAA    1358
GAGGAGTAGC AAAAGTAAAA GCTAGAATGA GATTGAATTC TGAGTCGAAA TACAAAATTT    1418
TACATATTCT GTTTCTCTCT TTTTCCCCCT CTTAG   CT GAA GAT GAT G   GTAAA    1470
                                        Ala Glu Asp Asp Glu
                                        -10
GTAGAAATGA ATTTATTTTT CTTTGCAAAC TAAGTATCTG CTTGAGACAC ATCTATCTCA    1530
GCATTGTCAG CTGAGGAAAA AAAAAAATGG TTCTCATGCT ACCAATCTGC CTTCAAAGAA    1590
ATGTGGACTC AGTAGCACAG CTTTGGAATG AAGATGATCA TAAGAGATAC AAAGAAGAAC    1650
CTCTAGCAAA AGATGCTTCT CTATGCCTTA AAAAATTCTC CAGCTCTTAG AATCTACAAA    1710
ATAGACTTTG CCTGTTTCAT TGGTCCTAAG ATTAGCATGA AGCCATGGAT TCTGTTGTAG    1770
GGGGAGCGTT GCATAGGAAA AAGGGATTGA AGCATTAGAA TTGTCCAAAA TCAGTAACAC    1830
CTCCTCTCAG AAATGCTTTG GGAAGAAGCC TGGAAGGTTC CGGGTTGGTG GTGGGGTGGG    1890
GCAGAAAATT CTGGAAGTAG AGGAGATAGG AATGGGTGGG GCAAGAAGAC CACATTCAGA    1950
GGCCAAAAGC TGAAAGAAAC CATGGCATTT ATGATGAATT CAGGGTAATT CAGAATGGAA    2010
GTAGAGTAGG AGTAGGAGAC TGGTGAGAGG AGCTAGAGTG ATAAACAGGG TGTAGAGCAA    2070
GACGTTCTCT CACCCCAAGA TGTGAAATTT GGACTTTATC TTGGAGATAA TAGGGTTAAT    2130
TAAGCACAAT ATGTATTAGC TAGGGTAAAG ATTAGTTTGT TGTAACAAAG ACATCCAAAG    2190
ATACAGTAGC TGAATAAGAT AGAGAATTTT TCTCTCAAAG AAAGTCTAAG TAGGCAGCTC    2250
AGAAGTAGTA TGGCTGGAAG CAACCTGATG ATATTGGGAC CCCCAACCTT CTTCAGTCTT    2310
GTACCCATCA TCCCCTAGTT GTTGATCTCA CTCACATAGT TGAAAATCAT CATACTTCCT    2370
GGGTTCATAT CCCAGTTATC AAGAAAGGGT CAAGAGAAGT CAGGCTCATT CCTTTCAAAG    2430
ACTCTAATTG GAAGTTAAAC ACATCAATCC CCCTCATATT CCATTGACTA GAATTTAATC    2490
ACATGGCCAC ACCAAGTGCA AGGAAATCTG GAAAATATAA TCTTTATTCC AGGTAGCCAT    2550
ATGACTCTTT AAAATTCAGA AATAATATAT TTTTAAAATA TCATTCTGGC TTTGGTATAA    2610
AGAATTGATG GTGTGGGGTG AGGAGGCCAA AATTAAGGGT TGAGAGCCTA TTATTTTAGT    2670
TATTACAAGA AATGATGGTG TCATGAATTA AGGTAGACAT AGGGGAGTGC TGATGAGGAG    2730
CTGTGAATGG ATTTTAGAAA CACTTGAGAG AATCAATAGG ACATGATTTA GGGTTGGATT    2790
TGGAAAGGAG AAGAAAGTAG AAAAGATGAT GCCTACATTT TTCACTTAGG CAATTTGTAC    2850
CATTCAGTGA AATAGGGAAC ACAGGAGGAA GAGCAGGTTT TGGTGTATAC AAAGAGGAGG    2910
ATGGATGACG CATTTCGTTT TGGATCTGAG ATGTCTGTGG AACGTCCTAG TGGAGATGTC    2970
CACAAACTCT TCTACATGTG GTTCTGAGTT CAGGACACAG ATTTGGGCTG GAGATAGAGA    3030
TATTGTAGGC TTATACATAG AAATGGCATT TGAATCTATA GAGATAAAAA GACACATCAG    3090
AGGAAATGTG TAAAGTGAGA GAGGAAAAGC CAAGTACTGT GCTGGGGGGA ATACCTACAT    3150
TTAAAGGATG CAGTAGAAAG AAGCTAATAA ACAACAGAGA GCAGACTAAC CAAAAGGGGA    3210
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GAAGAAAAAC CAAGAGAATT CCACCGACTC CCAGGAGAGC ATTTCAAGAT TGAGGGGATA   3270
GGTGTTGTGT TGAATTTTGC AGCCTTGAGA ATCAAGGGCC AGAACACAGC TTTTAGATTT   3330
AGCAACAAGG AGTTTGGTGA TCTCAGTGAA AGCAGCTTGA TGGTGAAATG GAGGCAGAGG   3390
CAGATTGCAA TGAGTGAAAC AGTGAATGGG AAGTGAAGAA ATGATACAGA TAATTCTTGC   3450
TAAAAGCTTG GCTGTTAAAA GGAGGAGAGA AACAAGACTA GCTGCAAAGT GAGATTGGGT   3510
TGATGGAGCA GTTTTAAATC TCAAAATAAA GAGCTTTGTG CTTTTTTGAT TATGAAAATA   3570
ATGTGTTAAT TGTAACTAAT TGAGGCAATG AAAAAAGATA ATAATATGAA AGATAAAAAT   3630
ATAAAAACCA CCCAGAAATA ATGATAGCTA CCATTTTGAT ACAATATTTC TACACTCCTT   3690
TCTATGTATA TATACAGACA CAGAAATGCT TATATTTTTA TTAAAAGGGA TTGTACTATA   3750
CCTAAGCTGC TTTTTCTAGT TAGTGATATA TATGGACATC TCTCCATGGC AACGAGTAAT   3810
TGCAGTTATA TTAAGTTCAT GATATTTCAC AATAAGGGCA TATCTTTGCC CTTTTTATTT   3870
AATCAATTCT TAATTGGTGA ATGTTTGTTT CCAGTTTGTT GTTGTTATTA ACAATGTTCC   3930
CATAAGCATT CCTGTACACC AATGTTCACA CATTTGTCTG ATTTTTTCTT CAGGATAAAA   3990
CCCAGGAGGT AGAATTGCTG GGTTGATAGA AGAGAAAGGA TGATTGCCAA ATTAAAGCTT   4050
CAGTAGAGGG TACATGCCGA GCACAAATGG GATCAGCCCT AGATACCAGA AATGGCACTT   4110
TCTCATTTCC CCTTGGGACA AAAGGGAGAG AGGCAATAAC TGTGCTGCCA GAGTTAAATT   4170
TGTACGTGGA GTAGCAGGAA ATCATTTGCT GAAAATGAAA ACAGAGATGA TGTTGTAGAG   4230
GTCCTGAAGA GAGCAAAGAA AATTTGAAAT TGCGGCTATC AGCTATGGAA GAGAGTGCTG   4290
AACTGGAAAA CAAAAGAAGT ATTGACAATT GGTATGCTTG TAATGGCACC GATTTGAACG   4350
CTTGTGCCAT TGTTCACCAG CAGCACTCAG CAGCCAAGTT TGGAGTTTTG TAGCAGAAAG   4410
ACAAATAAGT TAGGGATTTA ATATCCTGGC CAAATGGTAG ACAAAATGAA CTCTGAGATC   4470
CAGCTGCACA GGGAAGGAAG GGAAGACGGG AAGAGGTTAG ATAGGAAATA CAAGAGTCAG   4530
GAGACTGGAA GATGTTGTGA TATTTAAGAA CACATAGAGT TGGAGTAAAA GTGTAAGAAA   4590
ACTAGAAGGG TAAGAGACCG GTCAGAAAGT AGGCTATTTG AAGTTAACAC TTCAGAGGCA   4650
GAGTAGTTCT GAATGGTAAC AAGAAATTGA GTGTGCCTTT GAGAGTAGGT TAAAAAACAA   4710
TAGGCAACTT TATTGTAGCT ACTTCTGGAA CAGAAGATTG TCATTAATAG TTTTAGAAAA   4770
CTAAAATATA TAGCATACTT ATTTGTCAAT TAACAAAGAA ACTATGTATT TTTAAATGAG   4830
ATTTAATGTT TATTGTAG  AA AAC CTG GAA TCA GAT TAC TTT GGC AAG CTT      4880
                    Glu Asn Leu Glu Ser Asp Tyr Phe Gly Lys Leu
                     -5                  1                   5
GAA TCT AAA TTA TCA GTC ATA AGA AAT TTG AAT GAC CAA GTT CTC TTC      4928
Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn Asp Gln Val Leu Phe
               10                  15                  20
ATT GAC CAA GGA AAT CGG CCT CTA TTT GAA GAT ATG ACT GAT TCT GAC      4976
Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp Met Thr Asp Ser Asp
          25                  30                  35
TGT AGA G   GTATTTTTT TTAATTCGCA AACATAGAAA TGACTAGCTA CTTCTTCCCA   5032
Cys Arg Asp
        40
TTCTGTTTTA CTGCTTACAT TGTTCCGTGC TAGTCCCAAT CCTCAGATGA AAAGTCACAG   5092
GAGTGACAAT AATTTCACTT ACAGGAAACT TTATAAGGCA TCCACGTTTT TTAGTTGGGG   5152
TAAAAAATTG GATACAATAA GACATTGCTA GGGGTCATGC CTCTCTGAGC CTGCCTTTGA   5212
ATCACCAATC CCTTTATTGT GATTGCATTA ACTGTTTAAA ACCTCTATAG TTGGATGCTT   5272
AATCCCTGCT TGTTACAGCT GAAAATGCTG ATAGTTTACC AGGTGTGGTG GCATCTATCT   5332
GTAATCCTAG CTACTTGGGA GGCTCAAGCA GGAGGATTGC TTGAGGCCAG GACTTTGAGG   5392
CTGTAGTACA CTGTGATCGT ACCTGTGAAT AGCCACTGCA CTCCAGCCTG GGTGATATAC   5452
AGACCTTGTC TCTAAAATTA AAAAAAAAAA AAAAAAAAAC CTTAGGAAAG GAAATTGATC   5512
AAGTCTACTG TGCCTTCCAA AACATGAATT CCAAATATCA AAGTTAGGCT GAGTTGAAGC   5572
AGTGAATGTG CATTCTTTAA AAATACTGAA TACTTACCTT AACATATATT TTAAATATTT   5632
TATTTAGCAT TTAAAAGTTA AAAACAATCT TTTAGAATTC ATATCTTTAA AATACTCAAA   5692
AAAGTTGCAG CGTGTGTGTT GTAATACACA TTAAACTGTG GGGTTGTTTG TTTGTTTGAG   5752
ATGCAGTTTC ACTCTGTCAC CCAGGCTGAA GTGCAGTGCA GTGCAGTGGT GTGATCTCGG   5812
CTCACTACAA CCTCCACCTC CCACGTTCAA GCGATTCTCA TGCCTCAGTC TCCCGAGTAG   5872
GTGGGATTAC AGGCATGCAC CACTTACACC CGGCTAATTT TTGTATTTTT AGTAGAGCTG   5932
GGGTTTCACC ATGTTGGCCA GGCTGGTCTC AAACCCCTAA CCTCAAGTGA TCTGCCTGCC   5992
TCAGCCTCCC AAACAAACAA ACAACCCCAC AGTTTAATAT GTGTTACAAC ACACATGCTG   6052
CAACTTTTAT GAGTATTTTA ATGATATAGA TTATAAAAGG TTGTTTTTAA CTTTTAAATG   6112
CTGGGATTAC AGGCATGAGC CACTGTGCCA GGCCTGAACT GTGTTTTTAA AAATGTCTGA   6172
CCAGCTGTAC ATAGTCTCCT GCAGACTGGC CAAGTCTCAA AGTGGGAACA GGTGTATTAA   6232
GGACTATCCT TTGGTTAAAT TTCCGCAAAT GTTCCTGTGC AAGAATTCTT CTAACTAGAG   6292
TTCTCATTTA TTATATTTAT TTCAG  AT AAT GCA CCC CGG ACC ATA TTT ATT      6343
                          Asp Asn Ala Pro Arg Thr Ile Phe Ile
                          40                  45
ATA AGT ATG TAT AAA GAT AGC CAG CCT AGA GGT ATG GCT GTA ACT ATC      6391
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
TCT GTG AAG TGT GAG AAA ATT TCA ACT CTC TCC TGT GAG AAC AAA ATT      6439
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Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
ATT TCC TTT AAG GTAAG ACTGAGCCTT ACTTTGTTTT CAATCATGTT AATATAATCA    6496
Ile Ser Phe Lys
ATATAATTAG AAATATAACA TTATTTCTAA TGTTAATATA AGTAATGTAA TTAGAAAACT    6556
CAAATATCCT CAGACCAACC TTTTGTCTAG AACAGAAATA ACAAGAAGCA GAGAACCATT    6616
AAAGTGAATA CTTACTAAAA ATTATCAAAC TCTTTACCTA TTGTGATAAT GATGGTTTTT    6676
CTGAGCCTGT CACAGGGGAA GAGGAGATAC AACACTTGTT TTATGACCTG CATCTCCTGA    6736
ACAATCAGTC TTTATACAAA TAATAATGTA GAATACATAT GTGAGTTATA CATTTAAGAA    6796
TAACATGTGA CTTTCCAGAA TGAGTTCTGC TATGAAGAAT GAAGCTAATT ATCCTTCTAT    6856
ATTTCTACAC CTTTGTAAAT TATGATAATA TTTTAATCCC TAGTTGTTTT GTTGCTGATC    6916
CTTAGCCTAA GTCTTAGACA CAAGCTTCAG CTTCCAGTTG ATGTATGTTA TTTTTAATGT    6976
TAATCTAATT GAATAAAAGT TATGAGATCA GCTGTAAAAG TAATGCTATA ATTATCTTCA    7036
AGCCAGGTAT AAAGTATTTC TGGCCTCTAC TTTTTCTCTA TTATTCTCCA TTATTATTCT    7096
CTATTATTTT TCTCTATTTC CTCCATTATT GTTAGATAAA CCACAATTAA CTATAGCTAC    7156
AGACTGAGCC AGTAAGAGTA GCCAGGGATG CTTACAAATT GGCAATGCTT CAGAGGAGAA    7216
TTCCATGTCA TGAAGACTCT TTTTGAGTGG AGATTTGCCA ATAAATATCC GCTTTCATGC    7276
CCACCCAGTC CCCACTGAAA GACAGTTAGG ATATGACCTT AGTGAAGGTA CCAAGGGGCA    7336
ACTTGGTAGG GAGAAAAAAG CCACTCTAAA ATATAATCCA AGTAAGAACA GTGCATATGC    7396
AACAGATACA GCCCCCAGAC AAATCCCTCA GCTATCTCCC TCCAACCAGA GTGCCACCCC    7456
TTCAGGTGAC AATTTGGAGT CCCCATTCTA GACCTGACAG GCAGCTTAGT TATCAAAATA    7516
GCATAAGAGG CCTGGGATGG AAGGGTAGGG TGGAAAGGGT TAAGCATGCT GTTACTGAAC    7576
AACATAATTA GAAGGGAAGG AGATGGCCAA GCTCAAGCTA TGTGGGATAG AGGAAAACTC    7636
AGCTGCAGAG GCAGATTCAG AAACTGGGAT AAGTCCGAAC CTACAGGTGG ATTCTTGTTG    7696
AGGGAGACTG GTGAAAATGT TAAGAAGATG GAAATAATGC TTGGCACTTA GTAGGAACTG    7756
GGCAAATCCA TATTTGGGGG AGCCTGAAGT TTATTCAATT TTGATGGCCC TTTTAAATAA    7816
AAAGAATGTG GCTGGGCGTG GTGGCTCACA CCTGTAATCC CAGCACTTTG GGAGGCCGAG    7876
GGGGGCGGAT CACCTGAAGT CAGGAGTTCA AGACCAGCCT GACCAACATG GAGAAACCCC    7936
ATCTCTACTA AAAATACAAA ATTAGCTGGG CGTGGTGGCA TATGCCTGTA ATCCCAGCTA    7996
CTCGGGAGGC TGAGGCAGGA GAATCTTTTG AACCCGGGAG GCAGAGGTTG CGATGAGCCT    8056
AGATCGTGCC ATTGCACTCC AGCCTGGGCA ACAAGAGCAA AACTCGGTCT CAAAAAAAAA    8116
AAAAAAAAAG TGAAATTAAC CAAAGGCATT AGCTTAATAA TTTAATACTG TTTTTAAGTA    8176
GGGCGGGGGG TGGCTGGAAG AGATCTGTGT AAATGAGGGA ATCTGACATT TAAGCTTCAT    8236
CAGCATCATA GCAAATCTGC TTCTGGAAGG AACTCAATAA ATATTAGTTG GAGGGGGGGA    8296
GAGAGTGAGG GGTGGACTAG GACCAGTTTT AGCCCTTGTC TTTAATCCCT TTTCCTGCCA    8356
CTAATAAGGA TCTTAGCAGT GGTTATAAAA GTGGCCTAGG TTCTAGATAA TAAGATACAA    8416
CAGGCCAGGC ACAGTGGCTC ATGCCTATAA TCCCAGCACT TTGGGAGGGC AAGGCGAGTG    8476
TCTCACTTGA GATCAGGAGT TCAAGACCAG CCTGGCCAGC ATGGCGATAC TCTGTCTCTA    8536
CTAAAAAAAA TACAAAAATT AGCCAGGCAT GGTGGCATGC ACCTGTAATC CCAGCTACTC    8596
GTGAGCCTGA GGCAGAAGAA TCGCTTGAAA CCAGGAGGTG TAGGCTGCAG TGAGCTGAGA    8656
TCGCACCACT GCACTCCAGC CTGGGCGACA GAATGAGACT TTGTCTCAAA AAAAGAAAAA    8716
GATACAACAG GCTACCCTTA TGTGCTCACC TTTCACTGTT GATTACTAGC TATAAAGTCC    8776
TATAAAGTTC TTTGGTCAAG AACCTTGACA ACACTAAGAG GGATTTGCTT TGAGAGGTTA    8836
CTGTCAGAGT CTGTTTCATA TATATACATA TACATGTATA TATGTATCTA TATCCAGGCT    8896
TGGCCAGGGT TCCCTCAGAC TTTCCAGTGC ACTTGGGAGA TGTTAGGTCA ATATCAACTT    8956
TCCCTGGATT CAGATTCAAC CCCTTCTGAT GTAAAAAAAA AAAAAAAAAA GAAAGAAATC    9016
CCTTTCCCCT TGGAGCACTC AAGTTTCACC AGGTGGGGCT TTCCAAGTTG GGGGTTCTCC    9076
AAGGTCATTG GGATTGCTTT CACATCCATT TGCTATGTAC CTTCCCTATG ATGGCTGGGA    9136
GTGGTCAACA TCAAAACTAG GAAAGCTACT GCCCAAGGAT GTCCTTACCT CTATTCTGAA    9196
ATGTGCAATA AGTGTGATTA AAGAGATTGC CTGTTCTACC TATCCACACT CTCGCTTTCA    9256
ACTGTAACTT TCTTTTTTTC TTTTTTTCTT TTTTTCTTTT TTTTTGAAAC GGAGTCTCGC    9316
TCTGTCGCCC AGGCTAGAGT GCAGTGGCAC GATCTCAGCT CACTGCAAGC TCTGCCTCCC    9376
GGGTTCACGC CATTCTCCTG CCTCACCCTC CCAAGCAGCT GGGACTACAG GCGCCTGCCA    9436
CCATGCCCAG CTAATTTTTT GTATTTTTAG TAGAGACGGG GTTTCACCGT GTTAGCCAGG    9496
ATGGTCTCGA TCTCCTGAAC TTGTGATCCG CCCGCCTCAG CCTCCCAAAG TGCTGGGATT    9556
ACAGGCGTGA GCCATCGCAC CCGGCTCAAC TGTAACTTTC TATACTGGTT CATCTTCCCC    9616
TGTAATGTTA CTAGAGCTTT TGAAGTTTTG GCTATGGATT ATTTCTCATT TATACATTAG    9676
ATTTCAGATT AGTTCCAAAT TGATGCCCAC AGCTTAGGGT CTCTTCCTAA ATTGTATATT    9736
GTAGACAGCT GCAGAAGTGG GTGCCAATAG GGGAACTAGT TTATACTTTC ATCAACTTAG    9796
GACCCACACT TGTTGATAAA GAACAAAGGT CAAGAGTTAT GACTACTGAT TCCACAACTG    9856
ATTGAGAAGT TGGAGATAAC CCCGTGACCT CTGCCATCCA GAGTCTTTCA GGCATCTTTG    9916
AAGGATGAAG AAATGCTATT TTAATTTTGG AGGTTTCTCT ATCAGTGCTT AGGATCATGG    9976
GAATCTGTGC TGCCATGAGG CCAAAATTAA GTCCAAAACA TCTACTGGTT CCAGGATTAA   10036
CATGGAAGAA CCTTAGGTGG TGCCCACATG TTCTGATCCA TCCTGCAAAA TAGACATGCT   10096
GCACTAACAG GAAAAGTGCA GGCAGCACTA CCAGTTGGAT AACCTGCAAG ATTATAGTTT   10156
CAAGTAATCT AACCATTTCT CACAAGGCCC TATTCTGTGA CTGAAACATA CAAGAATCTG   10216
CATTTGGCCT TCTAAGGCAG GGCCCAGCCA AGGAGACCAT ATTCAGGACA GAAATTCAAG   10276
```

```
ACTACTATGG AACTGGAGTG CTTGGCAGGG AAGACAGAGT CAAGGACTGC CAACTGAGCC  10336
AATACAGCAG GCTTACACAG GAACCCAGGG CCTAGCCCTA CAACAATTAT TGGGTCTATT  10396
CACTGTAAGT TTTAATTTCA GGCTCCACTG AAAGAGTAAG CTAAGATTCC TGGCACTTTC  10456
TGTCTCTCTC ACAGTTGGCT CAGAAATGAG AACTGGTCAG GCCAGGCATG GTGGCTTACA  10516
CCTGGAATCC CAGCACTTTG GGAGGCCGAA GTGGGAGGGT CACTTGAGGC CAGGAGTTCA  10576
GGACCAGCTT AGGCAACAAA GTGAGATACC CCCTGACCCC TTCTCTACAA AAATAAATTT  10636
TAAAAATTAG CCAAATGTGG TGGTGTATAC TTACAGTCCC AGCTACTCAG GAGGCTGAGG  10696
CAGGGGGATT GCTTGAGCCC AGGAATTCAA GGCTGCAGTG AGCTATGATT TCACCACTGC  10756
ACTTCTGGCT GGGCAACAGA GCGAGACCCT GTCTCAAAGC AAAAAGAAAA AGAAACTAGA  10816
ACTAGCCTAA GTTTGTGGGA GGAGGTCATC ATCGTCTTTA GCCGTGAATG GTTATTATAG  10876
AGGACAGAAA TTGACATTAG CCCAAAAAGC TTGTGGTCTT TGCTGGAACT CTACTTAATC  10936
TTGAGCAAAT GTGGACACCA CTCAATGGGA GAGGAGAGAA GTAAGCTGTT TGATGTATAG  10996
GGGAAAACTA GAGGCCTGGA ACTGAATATG CATCCCATGA CAGGGAGAAT AGGAGATTCG  11056
GAGTTAAGAA GGAGAGGAGG TCAGTACTGC TGTTCAGAGA TTTTTTTTAT GTAACTCTTG  11116
AGAAGCAAAA CTACTTTTGT TCTGTTTGGT AATATACTTC AAAACAAACT TCATATATTC  11176
AAATTGTTCA TGTCCTGAAA TAATTAGGTA ATGTTTTTTT CTCTATAG GAA ATG AAT   11233
                                                    Glu Met Asn
                                                    85

CCT CCT GAT AAC ATC AAG GAT ACA AAA AGT GAC ATC ATA TTC TTT CAG    11281
Pro Pro Asp Asn Ile Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Glu
        90                  95                  100
AGA AGT GTC CCA GGA CAT GAT AAT AAG ATG CAA TTT GAA TCT TCA TCA    11329
Arg Ser Val Pro Gly His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser
    105                 110                 115
TAC GAA GGA TAC TTT CTA GCT TGT GAA AAA GAG AGA GAC CTT TTT AAA    11377
Tyr Glu Gly Tyr Phe Leu Ala Cys Glu Lys Glu Arg Asp Leu Phe Lys
120                 125                 130                 135
CTC ATT TTG AAA AAA GAG GAT GAA TTG GGG GAT AGA TCT ATA ATG TTC    11425
Leu Ile Leu Lys Lys Glu Asp Glu Leu Gly Asp Arg Ser Ile Met Phe
                140                 145                 150
ACT GTT CAA AAC GAA GAC TAGCTATTAA AATTTCATGC C                    11464
Thr Val Gln Asn Glu Asp
            155
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 471 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: mouse
- (G) CELL TYPE: liver

(ix) FEATURE:
- (A) NAME/KEY: mat peptide
- (B) LOCATION: 1..471
- (C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
AAC TTT GGC CGA CTT CAC TGT ACA ACC GCA GTA ATA CGG AAT ATA AAT     48
Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1               5                   10                  15
GAC CAA GTT CTC TTC GTT GAC AAA AGA CAG CCT GTG TTC GAG GAT ATG     96
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
ACT GAT ATT GAT CAA AGT GCC AGT GAA CCC CAG ACC AGA CTG ATA ATA    144
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
TAC ATG TAC AAA GAC AGT GAA GTA AGA GGA CTG GCT GTG ACC CTC TCT    192
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
```

```
    50                  55                  60
GTG AAG GAT AGT AAA ATG TCT ACC CTC TCC TGT AAG AAC AAG ATC ATT   240
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
TCC TTT GAG GAA ATG GAT CCA CCT GAA AAT ATT GAT GAT ATA CAA AGT   288
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
GAT CTC ATA TTC TTT CAG AAA CGT GTT CCA GGA CAC AAC AAG ATG GAG   336
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
TTT GAA TCT TCA CTG TAT GAA GGA CAC TTT CTT GCT TGC CAA AAG GAA   384
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
        115                 120                 125
GAT GAT GCT TTC AAA CTC ATT CTG AAA AAA AAG GAT GAA AAT GGG GAT   432
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
AAA TCT GTA ATG TTC ACT CTC ACT AAC TTA CAT CAA AGT               471
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
Asn Phe Gly Arg Leu His Cys Thr Thr
1               5
```

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                   10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
         35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
     50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
 65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                 85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ser Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
Tyr Phe Gly Lys Leu Glu Ser Lys Leu Ser Val Ile Arg Asn Leu Asn
1               5                  10                  15
Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro Leu Phe Glu Asp
            20                  25                  30
Met Thr Asp Ser Asp Ser Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
        35                  40                  45
Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
    50                  55                  60
Ser Val Lys Ser Glu Lys Ile Ser Thr Leu Ser Ala Glu Asn Lys Ile
65                  70                  75                  80
Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
                85                  90                  95
Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
            100                 105                 110
Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu
        115                 120                 125
Lys Glu Arg Asp Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu
    130                 135                 140
Gly Asp Arg Ser Ile Met Phe Thr Val Gln Asn Glu Asp
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
Asn Phe Gly Arg Leu His Ala Thr Thr Ala Val Ile Arg Asn Ile Asn
```

```
1               5                   10                  15
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
    50                  55                  60
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Cys Gln Lys Glu
        115                 120                 125
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

```
Asn Phe Gly Arg Leu His Cys Thr Thr Ala Val Ile Arg Asn Ile Asn
1               5                   10                  15
Asp Gln Val Leu Phe Val Asp Lys Arg Gln Pro Val Phe Glu Asp Met
            20                  25                  30
Thr Asp Ile Asp Gln Ser Ala Ser Glu Pro Gln Thr Arg Leu Ile Ile
        35                  40                  45
Tyr Met Tyr Lys Asp Ser Glu Val Arg Gly Leu Ala Val Thr Leu Ser
    50                  55                  60
Val Lys Asp Ser Lys Met Ser Thr Leu Ser Cys Lys Asn Lys Ile Ile
65                  70                  75                  80
Ser Phe Glu Glu Met Asp Pro Pro Glu Asn Ile Asp Asp Ile Gln Ser
                85                  90                  95
Asp Leu Ile Phe Phe Gln Lys Arg Val Pro Gly His Asn Lys Met Glu
            100                 105                 110
Phe Glu Ser Ser Leu Tyr Glu Gly His Phe Leu Ala Ser Gln Lys Glu
        115                 120                 125
Asp Asp Ala Phe Lys Leu Ile Leu Lys Lys Lys Asp Glu Asn Gly Asp
    130                 135                 140
Lys Ser Val Met Phe Thr Leu Thr Asn Leu His Gln Ser
145                 150                 155
```